



Polymorphisms in the Gene That Encodes the Iron Transport Protein Ferroportin 1 Influence Susceptibility to Tuberculosis

Citation

Baker, Meghan Ann, Douglas Wilson, Kristina Wallengren, Andreas Sandgren, Oleg Iartchouk, Nisha Broodie, Sunali D. Goonesekra, Pardis C Sabeti, and Megan B. Murray. 2012. Polymorphisms in the gene that encodes the iron transport protein ferroportin 1 influence susceptibility to tuberculosis. *Journal of Infectious Disease* 205(7): 1043-1047.

Published Version

doi:10.1093/infdis/jis026

Permanent link

<http://nrs.harvard.edu/urn-3:HUL.InstRepos:9891821>

Terms of Use

This article was downloaded from Harvard University's DASH repository, and is made available under the terms and conditions applicable to Open Access Policy Articles, as set forth at <http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#OAP>

Share Your Story

The Harvard community has made this article openly available.
Please share how this access benefits you. [Submit a story](#).

[Accessibility](#)

**Polymorphisms in the gene encoding the iron transport protein ferroportin 1
influence susceptibility to tuberculosis**

Meghan A Baker^{1,2}, Douglas Wilson³, Kristina Wallengren^{2,4}, Andreas Sandgren², Oleg Iartchouk⁵, Nisha Broodie⁶, Sunali D Goonesekera², Pardis C Sabeti^{7,8},
Megan B Murray^{1,2,9}

¹ Division of Infectious Diseases, Massachusetts General Hospital, Boston, MA, US

² Department of Epidemiology, Harvard School of Public Health, Boston, MA, US

³ University of KwaZulu-Natal, Edendale Hospital, Pietermaritzburg, KwaZulu-Natal, South Africa

⁴ KwaZulu-Natal Research Institute for Tuberculosis and HIV, University of KwaZulu-Natal, Durban, KwaZulu-Natal, South Africa

⁵ Harvard Medical School, Partners Healthcare Center for Genetics and Genomics, Boston, MA, US

⁶ Columbia University College of Physicians and Surgeons, New York City, NY, US

⁷ Center for Systems Biology, Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA, US

⁸ Broad Institute of MIT and Harvard, Cambridge, MA, US

⁹ Division of Global Health Equity, Brigham & Women's Hospital, Boston, MA, US

Address for correspondence:

Megan B Murray, M.D., Sc.D.

Harvard School of Public Health, 641 Huntington Ave. Boston, MA 02115

Phone: 617-432-2781; Fax: 617-432-2565; Email: mmurray@hsph.harvard.edu

Running Head: *SLC40A1* SNPs influence TB susceptibility

Word Count: Abstract 101 words, Text 2003 words

Conflict of interest: All authors affirm that they have no financial or personal conflicts of interest.

Funding: This study was supported by the Milton Award from the Harvard Medical School (MM), an International Travel Award from the Harvard Initiative for Global Health (MB) and the Eleanor and Miles Shore 50th Anniversary Fellowship Program for Scholars in Medicine, Massachusetts General Hospital, Department of Medicine (MB).

Meetings: Data from this study was presented at the New England Tuberculosis Symposium, July, 2010, Cambridge, MA

Correspondence: Megan B Murray, M.D., Sc.D.

Harvard School of Public Health, 641 Huntington Ave. Boston, MA 02115

Phone: 617-432-2781; Fax: 617-432-2565; Email: mmurray@hsph.harvard.edu

Current affiliations:

Andreas Sandgren, Ph.D.: European Centre for Disease Prevention and Control, Stockholm, Sweden

Meghan A. Baker, M.D., Sc.D.: Department of Population Medicine, Harvard Medical School and Harvard Pilgrim Health Care Institute, Boston, MA USA

Abstract

Background: We studied the association between iron intake and polymorphisms in the iron transporter gene, *SLC40A1*, and the risk of tuberculosis.

Methods: We compared iron intake, the frequency of *SLC40A1* mutations, and interactions between these variables among 98 TB patients and 125 controls in Kwazulu-Natal, South Africa.

Results: Four *SLC40A1* SNPs were associated with an increased risk of tuberculosis and one with reduced risk. We also found a gene-environment interaction for four non-exonic SNPs and iron intake.

Conclusions: This pilot study demonstrated an association between polymorphisms in *SLC40A1* and tuberculosis and provided evidence for an interaction between dietary iron and *SLC40A1*.

Key words: *SLC40A1*, Ferroportin, FPN1, Tuberculosis, Mycobacterium, Iron, Genetic, Single nucleotide polymorphism (SNP), Gene-environment

One-third of the world's population is infected with *Mycobacterium tuberculosis* (*MTB*), but only a minority of these develop active tuberculosis. Excess iron has been implicated as a risk factor for TB in both in vitro and clinical studies. Restriction of iron in culture media curtails growth of *MTB*, and iron loading in mice enhances bacterial growth in lung and spleen [2]. Iron supplementation has been shown to lead to increased morbidity and mortality in patients while autopsies show that death from TB can be accompanied by splenic iron overload [3]. Recognizing the physiological importance of iron availability for mycobacterial growth [4], we hypothesized that functional variants of the iron transporter, ferroportin 1 (FPN1), may affect host susceptibility to TB.

“African iron overload syndrome” is characterized by increased iron and serum ferritin, but unlike “classic” HFE hemochromatosis, excess iron is localized in the reticulo-endothelial system. Although this syndrome had been ascribed to intake of traditional African beer brewed in non-galvanized steel drums, an autosomal dominant form of iron overload has been described in which iron accumulation is restricted to reticuloendothelial cells [6]; this entity is now believed to be identical to the “ferroportin disease” associated with polymorphisms in *SCL40A1*, the gene which encodes the iron exporter, FPN1 [5].

FPN1 is found on the basolateral surface of enterocytes and macrophage cell wall. The current model posits that dietary iron absorbed by enterocytes is moved into the circulation by FPN1, then circulates bound to transferrin and is delivered to tissues via endocytosis of the iron/transferrin complex. Most of the body's iron is used for hemoglobin production by erythrocytes, and iron from senescent red blood cells is recovered when macrophages ingest and catabolize these cells. Macrophages transport scavenged iron to bone marrow, where it is released via FPN1. Gain-of-function

mutations in *SCL40A1* prevent FPN-1 inactivation by the iron regulatory hormone hepcidin, and cause a classic hemochromatosis phenotype manifested by low macrophage iron, increased transferrin saturation, and hepatocellular iron loading [6]. Loss-of-function variants inhibit the protein from localizing to the cell surface, impairing iron egress and leading to macrophage iron overload [7].

To test the hypothesis that high levels of macrophage iron increase TB risk, we evaluated the gene-environment interactions between *SLC40A1* polymorphisms and dietary iron intake and outcome, TB disease.

Methods

We conducted this study in Pietermaritzburg in KwaZulu-Natal, South Africa, an area inhabited by the Zulu, a group at high risk for African iron overload syndrome [8, 27]. TB incidence in this community is high at 1,163 per 100,000 compared to overall South Africa incidence of 820 per 100,000 [28]. We enrolled consecutive HIV negative patients over 18 years old in whom pulmonary tuberculosis was confirmed by two consecutive sputum smears positive for acid fast bacilli. Controls were recruited from those who tested HIV negative at Voluntary Counseling and Testing centers and from households of cases. Controls were unrelated to cases, were >18 years old, and had no history or symptoms of TB disease. Covariates included gender, age, race, current smoking status, self-reported diabetes mellitus, current alcohol use, BCG vaccination and socio-economic status. To assess excess iron intake, we evaluated ingestion of home-brewed beer and vitamins. Host DNA was obtained from a saliva sample using the Oragene®DNA sample collection kit (DNA Genotek Inc, Kanata, ON, Canada) and was stored at -70°C.

The study was approved by the institutional review board of Harvard School of Public Health, the Biomedical Research Ethics Committee at the University of KwaZulu-Natal, Durban, and the Department of Health, KwaZulu-Natal, South Africa.

Sequencing of SLC40A1

To detect new polymorphisms specific to this population, we sequenced the flanking 5' and 3' regions, promoter regions, exons, and proximal non-coding regions of the *SLC40A1* gene from 28 cases and 18 controls. The primer design used Beckman Coulter Genomics' Linux-based amplicon design software. Genomic DNA was amplified using Thermo-StartReddyMix PCR Master Mix (ThermoScientific) and 0.2 uM forward and reverse PCR primers. Solid phase reversible immobilization chemistry (AMPure - Beckman Coulter Genomics) and dye-terminator fluorescent sequencing were used to purify the PCR products. Polymorphisms were detected using polyphred 5.04 (University of Washington).

Genotyping

We identified thirteen SNPs in the *SLC40A1* locus from the International HapMapProject dbSNP and selected 20 of the 41 polymorphisms identified by resequencing, choosing SNPs based on allele frequency > 2%, location in the coding regions, and SNP assay design parameters. SNP genotyping was performed using Sequenom MALDI-TOF primer extension assay using the Sequenom (San Diego, CA) MassARRAY system.

Statistical Analysis

Univariate analysis was conducted using logistic regression models in STATA version 10 . SNPs were excluded from the analysis if 1) they deviated from Hardy Weinberg equilibrium ($P < 0.001$), 2) $\geq 25\%$ of the data was missing, or 3) they had a pairwise

threshold of $r^2 \geq 0.8$ using linkage disequilibrium based pruning (PLINK 2007). Genotypes were coded using an allelic model, and allele frequency distributions were compared between cases and controls using PLINK. Gene-environment interactions with iron ingestion were assessed using the likelihood-ratio test (LRT) and a case-only analysis for a gene-environment interaction [8]. We considered results significant at the alpha = .05 level.

Results

Population

We identified 104 persons with TB and 127 controls, of whom 100 cases and 126 controls agreed to participate in the study. DNA samples were available from 98 cases and 125 controls.

Host characteristics

We found no association between iron ingestion through home-brewed beer, iron supplements, or multivitamins and TB disease. Factors found to be significantly associated with TB included age ($p < .001$), male sex ($p = .03$), and self-reported diabetes mellitus ($p = .01$).

Sequencing and genotyping

Resequencing identified 41 polymorphisms including 14 novel polymorphisms of which 33 were genotyped and 32 had a genotyping efficiency of $>75\%$.

SNP association analyses

We excluded one SNP which deviated from Hardy-Weinberg equilibrium and seven based on the linkage disequilibrium based pruning procedure. Table 2 shows that among the remaining 24 SNPs, four significantly increased the risk of TB disease (GRCh37190445284 $P=0.01$, 190445194 $P=0.05$, 190444943 $P=0.05$, 190423785 $P=0.04$, while one reduced risk (190442893 $P=0.03$). Three of these SNPs (GRCh37190445284, 190445194 and 190444943) occurred at low frequency while two (190423785 and 190442893) occurred in at least 25% of the population.

Gene-environment interactions

We found a significant gene-environment interaction between each of four SNPs and iron intake (GRCh37 190452872 LRT $P=0.02$, 190446541 LRT $P=.004$, 190432613 LRT $P=0.02$, 190423785 LRT $P=0.05$; Table 3). Among persons who consumed excess iron, two SNPs were associated with a statistically significant increased risk of TB disease, GRCh37 190446541, OR 3.33 (95% CI, 1.31, 8.48) and GRCh37 190423785, OR 3.19 (95% CI, 1.18, 8.61) and a third GRCh37 190452872 manifested an association that was not significant. Among those not exposed to increased iron, GRCh37 190432613 protected against TB disease (OR 0.51 (95% CI, 0.30, 0.87)). Three polymorphisms that were excluded by linkage disequilibrium based pruning, were closely associated with polymorphisms with evidence of gene-environment interactions: GRCh37 190452957 with GRCh37 190452872; and GRCh37 190428248 and 190425474 with GRCh37 190432613.

Of note, although GRCh37 190430096, causing the amino acid substitution Q248H, was not found to be associated with TB disease, the OR among participants with iron exposure was 0.13 (95% CI, 0.01, 2.49) compared to 1.15 (95% CI, 0.55, 2.41) in those without.

Discussion

Multiple lines of evidence suggest that host iron status influences both the occurrence and outcomes of tuberculosis. Here, we assessed the association between polymorphisms in the gene *SLC40A1* and active TB and found an association between TB disease and five polymorphisms within the iron transporter gene associated locus. Furthermore, we found preliminary evidence that iron ingestion through home-brewed beer and vitamin supplements modified the association between TB and four polymorphisms. These data suggest that iron metabolism defects may affect TB susceptibility and raise the possibility of a gene-environment interaction between *SLC40A1* and iron intake in modifying the risk of TB.

Mutations in *SLC40A1* could affect *MTB* growth and replication through multiple routes. Some *SLC40A1* SNPs alter the efficacy of FPN1 in absorbing iron across the duodenum and in iron export from macrophages [5], changes which may affect mycobacterial iron availability. *SLC40A1* mutants also manifest altered levels of pro-inflammatory cytokines including tumor necrosis factor- α and macrophage migration inhibitory factor [9] and FPN1 over-expression has been shown to impair nitric oxide production in *MTB* infected macrophages [10].

In this study, we identified SNPs associated with TB disease in the promoter, untranslated, intronic and downstream regions of *SLC40A1*. Transcriptional and translational control of *SLC40A1* expression is thought to be tightly regulated by iron, and polymorphisms in non-exonic regions of *SLC40A1* may alter such regulation (11). For example, Marro reported that heme upregulates *SLC40A1* transcription and that this activation relies on a highly conserved enhancer region in the promoter [12]. Deletion of

the iron responsive element (IRE) in the 5' UTR region abrogated this effect, suggesting that the IRE confers translational regulation through iron regulatory proteins that bind to the mRNA transcript in low iron states to prevent translation. Alternative promoters and splicing have also led to the loss of an IRE in *SLC40A1* transcripts in vivo [13]. Notably, two of the polymorphisms that we found to be associated with TB disease are located in the 5'UTR in proximity to the IRE, raising the possibilities that polymorphisms in the promoter regions may alter transcriptional regulation while those in the untranslated regions may affect the function of the IRE and therefore translational regulation.

Iron ingestion modified the association between TB disease and four SNPs found near the promoter, and in intronic and untranslated regions. This interaction may have resulted from altered iron absorption at the level of the duodenum or impaired iron egress from macrophages in the setting of excess iron intake. Polymorphisms affecting transcription may render *SLC40A1* unresponsive to iron regulation through the IRE, which may also lead to macrophage iron overload. We note that the mutation, Q248H has been previously associated with an iron metabolism phenotype in African and African Americans and that affected carriers exhibit elevated ferritin levels, reduced mean corpuscular volumes, elevated C-reactive protein levels, and low levels of tumor necrosis factor and macrophage inhibitory factor [14]. In vitro experiments show that Q248H mutants retain their ability to export iron, and some investigators have speculated that the mutation may produce a variant of FPN1 that is not inhibited by hepcidin and thus functions as a “permanently turned-on iron exporter” [15]. Our finding that Q248H was protective against TB disease among those with increased iron intake, although not statistically significant, is consistent with this phenotype, since carriers would be expected to have low macrophage iron levels even in the presence of excess circulating iron.

This study had multiple limitations. Although we attempted to capture relevant SNPs, we did not sequence the entire gene and may have missed non-exonic polymorphisms associated with TB disease. Since we did not directly measure host iron indices but assessed iron ingestion from vitamin supplements and home-brewed beer, we may have misclassified host iron status. Although previous reports indicate that the iron content of home-brewed beer is 258 fold higher than that found in commercial beer [15], we did not measure the iron content of the beer or systematically account for the frequency of beer intake. Finally, we did not test for the possibility of population stratification; however, we expect that bias would be minimal due to the homogenous study population with common Zulu ancestry.

This candidate gene study was planned as an exploratory pilot study and was not powered to account for multiple testing. However, the fact that 5 of 24 SNPS in the *SLC40A1*-associated locus were found to be associated with TB disease in a biologically plausible candidate gene suggests that this gene is a good candidate for further study. Future studies should sequence the entire *SLC40A1* gene to capture the full range of polymorphisms, include rigorous measurements of iron status and intake, carefully document covariates and be powered to assess potential gene-environment interactions. In addition, studies assessing the molecular mechanisms for the associations between *SLC40A1* polymorphisms and TB disease, including measurement and evaluation of *SLC40A1* transcripts, would complement the genetic studies and provide further insight into the function of FPN1.

Funding: This work was supported by the Milton Award from the Harvard Medical School (MM), an International Travel Award from the Harvard Initiative for Global Health (MB) and the Eleanor and Miles Shore 50th Anniversary Fellowship Program for Scholars in Medicine, Massachusetts General Hospital, Department of Medicine (MB).

Acknowledgements: We thank Alison Brown for her generous donation of time and resources for genotyping procedures. We also thank Fikelephi Sithole and Lindiwe Zondi for their work in the field interviewing patients and collecting samples. We appreciate biostatistical advice from Eric Tchetgen Tchetgen, Ph.D., Alkes Price, Ph.D. and Liming Liang, Ph.D.

Conflicts of Interest: All authors affirm that they have no financial or personal conflicts of interest.

References

1. Boelaert JR, Vandecasteele SJ, Appelberg R, Gordeuk VR. The effect of the host's iron status on tuberculosis. *J Infect Dis*. 2007 Jun 15; 195(12):1745-53.
2. Lounis N, Truffot-Pernot C, Grosset J, Gordeuk VR and Boelaert JR. Iron and *Mycobacterium tuberculosis* infection. *J Clin Virol* 2001;20:123-
3. Gordeuk VR, McLaren CE, MacPhail AP, Deichsel G and Bothwell The associations of iron overload in Africa with hepatocellular carcinoma and tuberculosis: Strachan's 1929 thesis revisited. *Blood* 1996;87:3470-6.
4. De Voss JJ, Rutter K, Schroeder BG, Su H, Zhu Y and Barry CE, 3rd. The salicylate-derived mycobactin siderophores of *Mycobacterium tuberculosis* are essential for growth in macrophages. *Proc Natl Acad Sci U S A* 2000;97:1252-75.
5. Gordeuk VR, Caleffi A, Corradini E, et al. Iron overload in Africans and African-Americans and a common mutation in the SCL40A1 (ferroportin 1) gene. *Blood Cells Mol Dis* 2003;31:299-304.
6. Pietrangelo A. The ferroportin disease. *Blood Cells Mol Dis* 2004;32:131-8.
7. Mayr R, Janecke AR, Schranz M, et al. Ferroportin disease: A systematic meta-analysis of clinical and molecular findings. *J Hepatology*;
8. VanderWeele TJ, Hernandez-Diaz S and Hernan MA. Case-only gene-environment interaction studies: when does association imply mechanistic interaction? *Genet Epidemiol*;34:327-34.
9. Kasvosve I, Debebe Z, Nekhai S and Gordeuk VR. Ferroportin (SLC40A1) Q248H mutation is associated with lower circulating plasma tumor necrosis factor-alpha and macrophage migration inhibitory factor concentrations in African children. *Clin Chim Acta*;411:1248-52.
10. Johnson EE, Sandgren A, Cherayil BJ, Murray M and Wessling-Resnick M. Role of ferroportin in macrophage-mediated immunity. *Infect Immun*;78:5099-106.
11. Marro S, Chiabrando D, Messana E, et al. Heme controls ferroportin1 (FPN1) transcription involving Bach1, Nrf2 and a MARE/ARE sequence motif at position -7007 of the FPN1 promoter. *Haematologica*;95:1261-8.
12. Galy B, Ferring-Appel D, Kaden S, Grone HJ and Hentze MW. Iron regulatory proteins are essential for intestinal function and control key iron absorption molecules in the duodenum. *Cell Metab* 2008;7:79-85.
13. Cianetti L, Segnalini P, Calzolari A, et al. Expression of alternative transcripts of ferroportin-1 during human erythroid differentiation. *Haematologica* 2005;90:1595-606.

14. Rivers CA, Barton JC, Gordeuk VR, Acton RT, Speechley MR, Snively BM, Leiendecker-Foster C, Press RD, Adams PC, McLaren GD, Dawkins FW, McLaren CE, Reboussin DM. Association of ferroportin Q248H polymorphism with elevated levels of serum ferritin in African Americans in the Hemochromatosis and Iron Overload Screening (HEIRS) Study. *Blood Cells Mol Dis.* 2007 May-Jun;38(3):247-52.
15. Matsha T, Brink L, van Rensburg S, Hon D, Lombard C and Erasmus R. Traditional home-brewed beer consumption and iron status in patients with esophageal cancer and healthy control subjects from Transkei, South Africa. *Nutr Cancer* 2006;56:67-73.

Table 1. SNPs in *SLC40A1* and the association with TB disease

Position GRCh37	Reference SNP ID Number (rs)	Region	Risk Allele Frequency			
			TB Case SNP/Total	TB Control SNP/Total	P Value	Odds ratio (95% CI ¹)
190452872	rs116496357	Upstream	10/196	14/248	0.80	0.90 (0.39, 2.07)
190452748	rs6706281	Upstream	82/196	91/246	0.30	1.23 (0.83, 1.80)
190450880	rs13012833	Upstream	10/196	10/244	0.62	1.26 (0.51, 3.09)
190449526	rs10188680	Upstream	91/194	110/242	0.76	1.06 (0.73, 1.55)
190446541	rs3811621	Upstream	86/194	113/242	0.62	0.91 (0.62, 1.33)
190446284	rs10202029	Upstream	45/194	58/244	0.89	0.97 (0.62, 1.51)
190445284	rs13008848	5'UTR	5/196	0/246	0.01	13.80 (0.76, 251.07) ²
190445194	rs1568351	5'UTR	3/196	0/250	0.05	8.92 (0.46, 173.78) ²
190444943*		Intron	5/196	1/250	0.05	6.52 (0.76, 56.26)
190444630	rs1439816	Intron	38/196	54/244	0.48	0.85 (0.53, 1.35)
190442893	rs1439814	Intron	38/194	71/250	0.03	0.61 (0.39, 0.96)
190441647	rs3792079	Intron	61/194	83/242	0.53	0.88 (0.59, 1.31)
190437572	rs11568344	Coding Leu-Leu Position 129	17/196	26/246	0.50	0.80 (0.42, 1.53)
190432613	rs10188230	Intron	35/192	60/244	0.11	0.68 (0.43, 1.09)
190432172	rs994226	Intron	60/194	60/242	0.15	1.36 (0.89, 2.07)
190430177	rs2304704	Coding Val-Val Position 221	20/196	29/244	0.58	0.84 (0.46, 1.54)
190430096	rs11568350	Coding Gln-His Position 248	15/196	21/246	0.74	0.89 (0.45, 1.77)
190428903		Coding Asp-Val Position 270	1/196	1/250	0.86	1.28 (0.08, 20.54)
190426315	rs61525883	3'UTR	16/194	25/246	0.49	0.79 (0.41, 1.53)
190425579*		3'UTR	2/168	0/208	0.11	6.19 (0.30, 129.75) ²
190425481*		3'UTR	4/196	10/250	0.24	0.5 (0.15, 1.62)
190424842	rs11884632	Downstream	42/192	56/244	0.79	0.94 (0.60, 1.48)
190423785	rs2352262	Downstream	56/196	50/246	0.04	1.57 (1.01, 2.43)
190423678*		Downstream	4/190	2/246	0.25	2.62 (0.48, 14.48)

¹. CI = confidence intervals

². * =Novel SNPs found through the sequencing procedure

³. OR and CI estimated using the Haldane odds ratio and confidence intervals as there were 0 SNPs in the control population

⁴. Codon position 3

⁵. Codon position 2

⁶. Codon position 2

Table 2. SNPs in *SLC40A1* with evidence of a gene-environment association

Position GRCh37	Reference SNP ID Number (rs)	Likelihood Ratio Test for Interaction	Case-only Test Method for Interaction	TB Case SNP/Total	TB Control SNP/Total	Risk Allele Frequency	
						P Value	OR (95% CI)
190452872	rs116496357	0.02	0.06				
	Iron exposure			4/34	1/50	0.06	6.53 (0.70, 61.24)
	No iron exposure			6/162	13/198	0.23	0.55 (0.20, 1.47)
190446541	rs3811621	0.004	0.03				
	Iron exposure			12/32	16/48	0.01	3.33 (1.31, 8.48)
	No iron exposure			66/162	97/194	0.08	0.69 (0.45, 1.05)
190432613	rs10188230	0.02	0.05				
	Iron exposure			10/32	8/48	0.13	2.27 (0.78, 6.60)
	No iron exposure			25/160	52/196	0.01	0.51 (0.30, 0.87)
190423785	rs2352262	0.05	0.10				
	Iron exposure			14/34	9/50	0.02	3.19 (1.18, 8.61)
	No iron exposure			42/162	41/196	0.26	1.32 (0.81, 2.16)